

Developmental Immunology and Potential Windows
of Vulnerability to Asthma

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[Slide 1] Good morning, I'm John Armstrong, I run this immunology consulting company in Rockville, Maryland, but we employ the services of immunologists and toxicologists all over the country.

I want to thank Mark Miller and the organizers for the meeting for inviting me to speak out here. It's because we just submitted a rough draft of a monograph on the assessment of immunotoxicology in children to the U.S. EPA a few weeks ago that I was invited out here.

We heard quite a bit yesterday about the role of the lung in the development of childhood asthma from Drs. Leikauf, Balmes and Tager. But, as these speakers noted, during different stages of development most of the data that's available indicate the occurrence of asthma in most children comes from alterations or disruption of immune maturation.

The talk we heard from Dr. Holladay a few minutes ago laid the foundation for understanding how the immune system can be affected by various environmental toxicants during different stages of development. As he pointed out, most of the data come from animal studies and not from human. What I'd like to do is elaborate on what is known about the role of the human immune system in the development of asthma with particular emphasis on time lines of human immunological development.

I'll end my talk by going one step further than Dr. Holladay and speculating wildly on potential windows of vulnerability by all sorts of different chemicals and drugs that are currently perhaps not tested for immunotoxicity.

[Slide 2] We know from yesterday's talks that asthma is an inflammatory disorder of unknown cause. We recognize three distinct components involving airway obstruction that resolve either spontaneously or after treatment; that there is hyper-responsiveness of the airways to a variety of stimuli, and that persistent inflammation of the airway is also a symptom of this disease.

From Dr. Goldman's talk we heard that the coughing and wheezing, chest tightness and shortness of breath associated with asthma affects more than 14 million Americans; a great deal of those are children. We know that asthma affects boys more than girls, women more than men, blacks more than whites, and urban children more than rural. But according to Dr. Goldman, many of these things could be attributed to poverty levels.

We also know that the risk factors for the development of childhood asthma include tobacco smoke, dust mites, animal dander, cockroaches, molds, pollens, cold air, food sulfites, some infections, some medicines, and genetic factors. We've heard that asthma becomes chronic and involves airway damage. And we've heard a little bit about the roles some cytokines played in the mechanism of induction of that damage.

It's clear that the airway responsiveness of asthma is related to serum IgE levels, and that suggests that it's the humoral arm of the immune system that's playing the biggest part there. But to understand how the humoral immune response can be elicited or altered by allergens or asthmagens one must know how the differentiation of peripheral T-cells can be deviated to help B-cells make IgE, as well as how T-cell subsets differentiate in the thymus in the first place.

So I'm going to review some basic immunology. I'm assuming most of you are not immunologists and I'm going to go through it slowly. B-cells can only make IgE to worsen asthma if they first get help from T-cells. And T-cells develop like B-cells, from pluripotent stem cells, like Steve Holladay just said. These pluripotent stem cells all originate in the bone marrow, whether they form B-cells or T-cells, during the postnatal period. But they also develop in the yolk sac and the fetal liver in the prenatal period.

[Slide 3] A T-cell progenitor cell enters the thymus via the subcapsular region of the thymus where it acquires surface expression of two major molecules that all immunologists know about, and that's CD4 and CD8. CD4 cells are those T-cells, which are eliminated in AIDs. CD8 cells you don't hear anything about in the literature, and that's because nobody's interested in basic immunology, they're interested in clinical immunology. But CD8s play a big role in clinical immunology.

So these nascent thymocytes, as they're called, when they enter the thymus are double positive: they express both CD4 and CD8. You don't see that in the periphery; you don't see that in neonates; you don't see that in grownups; you only see that in the thymus. But when T-cells leave the thymus they only express CD4 or CD8.

And how do they get there? A T-cell matures in two major steps. It goes through something called positive selection where it learns to recognize self-cells in the thymic cortex, this outer region of the thymic lobe, and it does that by recognizing molecules called major histocompatibility complex (MHC) molecules on the surface of cortical epithelial cells.

Ninety percent of those cells die by apoptosis if they do not recognize self-MHC molecules. So not only do you get a lot of apoptosis and a lot of cell death in the thymus, what that means is that you're also losing 90% of the potential T-cell antigen-binding repertoire, so there's potential here for skewing the antigen-binding repertoire of T-cells later on that enter the periphery. Any chemical that could interfere with the

development of the antigen-binding repertoire might be able to do so at this stage, by interfering with positive selection.

Then a T-cell migrates to the cortico-medullary junction and it goes through the second major process of T-cell education, and that's negative selection. Negative selection is where those T-cells not only recognize self-MHC but also do so too tightly such that they wouldn't want to let go. Merely by recognizing self-MHC, without any influence from outside foreign peptides of things that they're supposed to react against, if they recognize self-MHC too tightly they'll become activated and kill that self-cell. We don't want those in the periphery; that causes autoimmunity. It's the disruption of that step that Steve Holladay was talking about; any kind of chemical that interferes with negative selection could lead to autoimmunity.

So we destroy 90% of the cells at that step also, perhaps 99% of the cells at that step. That's great, it gets rid of autoreactive T-cells. It also skews the antigen-binding repertoire and it cuts it down a great deal. There is potential there for influence by environmental toxicants.

Mature T-cells then exit the thymus through the medulla, and when they exit they are either CD4-positive or CD8-positive. The CD4-positive T-cells react to immune cells that are out in the periphery, that is, to any cell that expresses MHC class II molecules.

CD8-positive cells react to any cell in the body, any nucleated cell, because all of those cells express class one molecules. CD8 cells generally differentiate into cytotoxic T-cells or CTL, and it's CTL that are responsible for this cell-mediated immunity, or CMI. Later on I'll talk about the effects of different environmental toxicants on suppressing CMI. That means we're suppressing the activation of CD8-positive T-cells, and it might be further upstream by inhibiting help from CD4-positive T-cells, but it could be just on the CD8s.

CD4-positive T-cells exit the thymus through the medulla also, but they have a much more ambiguous destiny. They don't just become a certain kind of cell that becomes activated and mediates cell-mediated immunity. CD4-positive T-cells can differentiate into two specific subtypes. CD4 cells are generally called helper cells and so we designate them with a T-H for helper; they can become TH1 or TH2.

The CD4-positive cells that exit the thymus, besides being called T-helper cells, because they can go to TH1 or TH2, are called TH0 cells. TH0 cells comprise all of the CD4-positive T-cells that exit the thymus. They are the only T-cells in a neonate. You might find a rare cell or two that's differentiated into TH1 or TH2, but for the most part neonates are born with TH0 cells, they're not TH1 or TH2. They are naive, they've never seen antigen, and they can't help anything, they can't help a single B-cell do anything.

The only way that a T-cell can help a B-cell to make antibody, for instance IgE, and cause asthma, at least IgE-mediated asthma, is first for that TH0 cell to get two signals. It has to see its cognate antigen. That is the peptide that it saw way back in the thymus when it was educated on what to recognize and what not to recognize. It has to see that out in the periphery and say, "oh, I see dioxin, I respond to that, I'm turned on". But it doesn't do that unless it also gets a second signal in the form of a certain cytokine. These are chemicals that antigen-presenting cells and other cells spew out in the midst of an immune response; those immune responses occur in secondary lymphoid organs.

So you've got thymocytes exiting the thymus, and they run all around the blood stream and occasionally enter a secondary lymphoid organ, like a lymph node or the spleen. It's only in those secondary lymphoid organs where immune responses to chemicals and pathogens occur.

[Slide 4] If a TH0 cell circulates through a secondary lymphoid organ and it sees its cognate antigen, and it receives a second signal in the form of

interleukin-12 it becomes a TH1 cell. Interleukin-12 comes from activated macrophages. Activated macrophages make IL-12 in response to intracellular pathogens like viruses and some bacteria.

You want to make a TH1 response to viruses and intracellular bacteria because TH1 responses activate cytotoxic T-cells and those are the cells that can kill cells that are infected with viruses and intracellular bacteria.

On the other hand, you have some bacteria that never enter a cell. These are extracellular bacteria and multi-cellular pathogens. We don't get rid of those with cytotoxic T-cells and cell-mediated immunity; we get rid of those with antibodies. That is the humoral arm of the immune response.

So in the center you see a TH0 cell, which exited the thymus. You see here it receiving interleukin-12 from an activated macrophage. The interleukin-12 drives the TH0 cell to become a TH1 CD4-positive T-cell. A TH1 cell helps a pre-CTL (pre-cytotoxic T lymphocyte) become an activated CTL by secreting an interleukin called IL-2, which used to be known as T-cell growth factor. This is the most important cytokine released by a T-helper-1 cell, interleukin-2. All T-cells make interleukin-2, they have to in order to grow, but TH1 cells make a lot more of it than TH2 cells.

If you are infected with an extracellular pathogen, there is an initial bolus release of interleukin-4 into your system, by mechanisms that are not yet clear. This can come from not yet well-characterized subsets of CD4-positive T-cells that express some natural killer cell markers, they release interleukin-4. Interleukin-4 can also come from mast cells, an important cell in asthma pathology.

In any case, when that circulating TH0 cell enters a lymph node it sees its cognate antigen, but as it's now in a bath of interleukin-4, it differentiates to become a TH2 cell.

A TH2 cell secretes a whole boat load of cytokines, including more interleukin-4, but also interleukin-5, -6, -9, -10, -13, transforming growth factor beta, all sorts of cytokines, and these cytokines help B-cells become activated.

A B-cell also has to see its cognate antigen in this secondary lymphoid organ. When a B-cell receives this chemokine signal, this cytokine signal, and it also sees its cognate antigen in a lymph node or spleen it forms what's called a germinal center. You know that a fetus or a neonate or an early child has been exposed to a barrage of pathogens or antigenic insults when you see the formation of germinal centers. Neonates are not born with germinal centers; they only get them after they're exposed to antigens in the environment.

When a B-cell makes antibodies that constitutes the humoral arm of the immune system. So whether you get humoral immunity and things cleared by antibodies or cell-mediated immunity and things cleared by CTLs depends on which way you swerve a TH0 cell towards.

Particularly of interest to immunologists studying asthma are some of the signals which are known to determine whether a B-cell secretes asthma-promoting IgE molecules as opposed to IgA or IgG because B-cells are born with IgM and IgD on their surfaces. They can switch then if they get the right signals.

For example, very low doses of antigen tend to make B-cells switch to IgE. Multi-cellular pathogens tend to make B-cells switch to IgE. And a lot of IL-4 coming from a TH2 cell makes a B-cell want to make IgE. But on the other hand, low levels of interleukin-4 released from T-helper-2 cells in the presence of other signals cause B-cells to switch to IgG or IgA. This might be the way to divert the response away from something that might promote asthma.

It would be very helpful if we as immunologists could modify any stimulus to direct T-helper-cell differentiation toward the type of immunity, humoral or cell-mediated, which would be of most benefit to a patient. We already know that once a

step has been taken towards TH1- or TH2-ness the cytokines produced inhibit the ability of the system to deviate in the other direction. A problem for an immunologist to solve in asthma might not be just to modulate IgE production in humoral immunity, but entirely to deviate the response away from a TH2 response all together and towards TH1.

As I said earlier, if the decision really is irreversible then prophylactic intervention might be a way to avert the inception of asthma. That's exactly what seems to be happening with some early childhood infections which drive the immune system to make a TH1 response.

And then some of you are thinking, well, what about vaccinations? As vaccinology becomes more sophisticated vaccines will be designed not only with the intent of clearing an infection, which is the only intent we have right now with vaccines, but in the future we will ensure that vaccines direct the clearance of a pathogen by the appropriate T-helper-cell differentiation pathway.

One possible answer to yesterday's question about, "Why in the face of a decline of cigarette smoking do we still see an increase in the prevalence of asthma in this country?", might be because of the success of childhood vaccination programs. There is a possibility that these vaccines are eliciting TH2 responses instead of TH1. Both responses might be protective, but one might increase the prevalence of asthma.

Dr. Tager mentioned yesterday a study by Prescott, et al., reporting that all children are born with a T-helper-2 profile. Why are children born with a T-helper-2 profile when a response towards environmental antigens gives them asthma? Why do we want that? We don't want asthma. But Dr. Tager explained that the placental environment ensures an in utero TH2 state.[Slide 5] But why TH2 if it's so closely associated with asthma and allergy? In fact, the intrauterine epithelium and trophoblast layers secrete lots of T-helper-2 cytokines, including IL-4 and IL-10. T-helper-2 cytokines in the periphery, and in an adult animal, as I mentioned a minute ago, shut down T-helper-1 differentiation. The fetus is promoting an immunosuppressive state in

the mother. TH1 responses lead to inflammation. If you have a TH1 response and you activate a cytotoxic T-cell you kill cells. You start killing cells and you get an inflammatory response, all sorts of inflammatory cells will infiltrate tissue. You get tissue damage. You can get rejection of the fetus. But the amniotic fluid surrounding a fetus also constitutively expresses a cytokine called interleukin-1. Amniotic interleukin-1 may also be suppressing in utero infection-induced inflammatory responses. While interleukin-1 is normally thought of as an inflammatory cytokine, it's one of those cytokines that activates the acute phase response. This is what you see in response to all sorts of infections. In the fetus at least it also induces a negative feedback regulatory pathway involving the hypothalamic pituitary adrenal axis. The result of that is the production of glucocorticoids, as you see here, which shut down any chance of inflammation. They do that by preventing the activation of macrophages that might otherwise become activated through stimulation by a bacteria or by some adjuvant (the goo you put a vaccine in).

An activated macrophage makes interleukin-1 and it makes TNF-alpha. TNF-alpha activates endothelial cells, which make interleukin-6, which stimulate the acute phase response and cause all sorts of inflammation. Possibly, resulting in the rejection of the fetus.

So the fetus is bathed in interleukin-1. Interleukin-1 constitutive expression could and does cause constitutive expression of glucocorticoids, which probably prevents any initial response to infection in the fetus by bacteria. It prevents an inflammatory response and its own rejection. As a result of that a neonate is born with this, in this bath of T-helper-2 cytokines and makes T-helper-2 responses to everything in the world when it's born.

Other factors also play a role in the immunosuppressive state of a fetus, and these include nonclassical MHC proteins, proteins called HLA-G and some other things, but we're not going to talk about that today.

Dr. Leikauf mentioned something yesterday about finding in the lung an up-regulated expression of metallothionein. That was a very interesting finding for me to hear. This was in response to nickel in occupational exposures. We know that metallothionein is found at elevated levels in mammalian fetal liver and neonatal thymus, and these are organs of intensely lymphoproliferative potential. We also know that metallothionein, besides binding and sequestering heavy metal cations and reactive toxicants, is up-regulated by several cytokines of the acute phase response and is capable of altering several aspects of lymphocyte function. So, when you talk about exposure to heavy metals you might also be talking about alteration of lymphoid differentiation and proliferation in the fetal liver and in the neonatal thymus.

Dr. Tager also mentioned that while non-atopic children switch towards a TH-1 response in the first two years of life. Atopic children fail to switch and strengthen their TH2 responses. In fact, the authors of that report reported that non-atopics begin to switch within the first year of life, and we have found data that switch to increased amounts of TH1-type cytokines occurs in the first month of life. So, if you're going to sensitize an infant to become atopic, if it has the genetic potential to do so, that sensitization may happen in the first weeks postnatally.

What is it in that first year of life that determines the direction of immune deviation besides genetics? It could be environmental exposures. It's probably both genetics and environment. There are some things we do know. I'll tell you what is known, but I'm going to speculate freely about what I think might be possible. I thought I was allowed to do that in this symposium. I'm not being very cautious, so write this down, a lot of this is speculation. When I say we know something we know it, you can quote me on that.

And I'm going to give you a lot of detailed time lines of immune development; I have sources for every statement I make. I'm not giving those to you in

writing today because we've submitted the draft to the EPA, it's their publication, we'll let them publish it and then you can have it.

[Slide 6] We have reports that chlordane is associated with immunomodulation, cancer in humans (A-L-L in children specifically), immunodeficiency in animals, and an alteration of cell-mediated immunity in developing animals. Remember CMI, or cell-mediated immunity, is the result of TH1 responses. A toddler has predominantly TH1 responses. So alteration could leave a child pretty immunosuppressed, or even predisposed to making TH2 responses.

Toxaphene has been shown to suppress humoral antibody responses, responses in developing animals, and immunosuppression in adult animals, although human data is lacking. Steve was right on the mark on this, there's just very little known and very few conclusions you can draw about which chemicals can do exactly what in humans.

Again, although human data is lacking, hexachlorobenzene has been shown to be immunosuppressive in adult animals, to depress CMI in developing animals, and enhance antibody responses in developing animals. HCB has also been shown to alter lymphocyte trafficking in developing animals. Lymphocyte trafficking is the process by which T- and B-cells can find a site of infection and fight it or can home to lymph nodes and spleen cells. When they are only naive cells they want to find the antigen in the first place and become activated and start an immune response. So if you alter lymphocyte trafficking that means you're cutting down on the ability for the adaptive immune response to take over and clear an infection and you're relying totally on your innate immune system. That can be effective in some cases, but in most cases it won't be enough.

In adult animals lead has been shown to alter CMI and humoral immunity end points, to increase the TH2/TH1 ratio by tenfold, and increase TH2 cytokine levels. In

prenatal and lactating animals lead has also been shown to modulate CMI end points, to suppress delayed-type hypersensitivity responses (DTH), and in prenatal animals alone, to increase IgE responses. We have inconsistent reports on lead in humans, but it seems that lead can cause CMI immunomodulations in adults and increased IGE production in children.

PCBs are known to cause transient alterations in CMI and humoral immunity in adults, although findings of immunosuppression in children have been contradictory. Dioxins are known to depress DTH responses in adults, as well as to be associated with non-Hodgkin's lymphoma and soft tissue sarcoma. In children dioxins cause changes in T-cell sub-populations, TH1, TH2, but the data is sparse and some of it's contradictory.

[Slide 7] To be able to predict what those kinds of effects might have on the developing immune system in children one needs to know when the different structural and functional components of the human immune system develop. I'll verbalize some of these for you but, again, I didn't give you these in a handout. You'll have those hopefully before the end of the year in a very nice publication by Dr. Firestone's office.

[Slide 8] During the first weeks of gestation stem cells are already found in the mesoderm of the yolk sac, but two months after conception they're in the fetal liver.

By 15 -- this is all human -- by 15 days of gestation Peyer's patches have formed. These are gut-associated lymphoid tissue along the intestine. At six weeks of gestation, hematopoietic cells migrate from the yolk sac to the fetal liver, and thymic tissue can be identified.

At six to seven weeks of gestation reticular mesenchymal cells aggregate, and these comprise the beginning of a spleen. You also see ossification, of formation of

bone, and subsequently the bone marrow, as you know a primary lymphoid organ where pluripotent stem cells form.

At 60 days' gestation thymocytes expressing known T-cell markers in the yolk sac are capable of colonizing the thymus.

Nine to 12 weeks' gestation B-cells produce immunoglobulin-M, you can already make an immune response; and this is in the first trimester.

At 13 weeks' gestation IgM and IgD are present in a B-cell, and that constitutes a mature B-cell. They can respond in every way that a postnatal B-cell can. You also see small populations of IgA and IgG at the end of the first trimester.

IgA and IgG only come about from secondary immune responses. The T-cells are capable but early gestational B cells generally don't make IgA and IgG. The most current literature says that the reason for that is because the antigen-presenting cells which provide help to a T-cell are not mature until later in gestation.

In the second and third trimesters, for instance from 15 to 20 weeks of gestation, we see mature CD4-positive, CD8-positive T-cell receptor expressing T-cells.

At 17 weeks gestation maternal antibodies enter the fetal circulation.

At 18 weeks gestation the fetal thymus resembles the thymus of a newborn, so we're barely into the second trimester and the thymus is ready.

At 30 weeks gestation you see IGA secreting plasma cells, so the gut-associated lymphoid tissue is ready to take off.

In the last four to six weeks of gestation maternal antibodies are at their highest levels. They're actually lower when you're born than a six-week preemie has.

[Slide 9] At birth, the neonatal period -- that's my daughter Eloise -- hematopoietic cells are functionally mature, B-cells are in the bone marrow, secondary organs have B-cell zones. Mucosal-associated lymphoid tissues have follicles a few days after birth.

Maternal antibodies decline over the next six months.

We already talked about how TH1 switches to TH2, unless you're going to become atopic.

From birth to one month you see a progressive increase in the number of T-cells capable of producing IL-2 and gamma interferon. This is how we know that switch, that sensitization to become atopic can occur in the first month of life.

A nursing infant is developing critical components of the immune system. Pesticide exposure at this stage via breast milk could significantly impact development. IgG, IgA, IgE production is possible from neonatal B-cells at this stage. A few days after birth germinal centers appear.

From one to three months we see the development of the lytic activity of complement components. You're able to fight infections with antibodies and with cell-mediated immunity, but you also need your innate immune system, and a lot of the innate immune system depends on these proteins that make up complement. They work with antibodies to become part of the humoral immune system or without antibodies. Without them you're immuno-compromised; they don't develop, they're not there when you're born. There are different kinds of complement activity that can develop throughout your life. The classical pathway of complement develops only after one to three months.

Gamma-interferon production can reach adult levels only after a month and a half of life.

[Slide 10] It's not enough to say, when you're trying to determine what's important for risk assessment, that children are not just little adults. Actually this two-year-old you see is not just a little three-year-old, there are significant immune components to develop between two and three years of age. A neonate is not a little one-year-old immunologically speaking. A first-trimester fetus is not a late-trimester fetus.

It's important to keep these things in mind because it's not just that a lower amount of toxicant will have the same effect in an earlier development stage, but it'll

have a completely different effect because immune system components are not even there yet in some cases.

And then, as Steve Holladay just emphasized, we really don't know a lot about human windows of vulnerability to specific toxicants. So here's where in some cases I'll speculate.

[Slide 11] In the fetus the thymus begins to develop in the first trimester. Prenatal PCB and dioxin exposure may contribute to thymic atrophy. We just saw that DES can.

Prenatal exposure to benzo[a]pyrene alters development of the B-cell repertoire, we know that. It suppresses B-cell lymphopoiesis. Potentially that could happen at any time after B-lymphopoiesis first begins, early in the first trimester.

Germinal centers begin to develop in secondary lymphoid organs, so any inhibitors of repertoire development, and that means anything happening in germinal centers, may alter germinal center formation and the ability to mount a memory response. And that's in the neonatal period.

Prenatal and lactation exposure to toxaphene suppresses antibody responses in rodents. Well, antibody responses are humoral immunity. Any delayed development of that could lead to severe immunosuppression.

Neonates also have immature enzyme detoxification systems, the P-450 enzymes. I studied these in my first post-doc at Sandoz in Switzerland. So body burdens of similar exposure levels as in adults could be increased due to slower elimination of toxins.

Toxaphene also depresses macrophage functions in developing animals. If you depress macrophage functions you depress the ability to move towards TH1 response and clear intracellular pathogens.

What is the result of that? An intracellular pathogen, for instance, is a virus. Any kind of chemical that might suppress cell-mediated immunity in the neonatal period exposes an infant to a potential danger of a higher viral burden.

There are several examples of cancers that have a viral etiology. Nobody has looked at an increased viral burden in the neonatal period and its outcome in later life in cancer end points. That needs to be looked at. It's certainly a reasonable connection.

Hexachlorobenzene inhibits adhesion molecule-dependent trafficking. I talked about that potential impact. That's in early childhood.

Elevated lead levels in school-age children are associated with elevated IGE levels. That could increase our susceptibility to atopic and autoimmune disease in children and adults and also in early childhood (over 80% of asthmatics develop the disease prior to age three).

[Slide 12] I would like to acknowledge the people that have helped us put this report together. In particular Dr. David Chen, who couldn't make it to the conference this week, at the Office of Children's Health Protection, U.S. EPA. And then the Immunarm team, Ramona Leibnitz is my wife and a good immunologist. Deborah Loer-Martin's my co-P.I. of Loer-Martin and Associates in Ft. Myers, Florida. Other immunologists around the country I've worked with before on other projects worked on putting this report together: Evan Hermel in Vallejo, California; Heather DeGrendele in Dallas; Julie Lovchik in Maryland; Donna Roscoe in Maryland; our science illustrators, Jeff Aarons and my son Charles Armstrong at the Maryland Institute College of Art.

And, so I thank you. (Applause.)